Targeted Gene Removal

The invention relates to a method of removing a selectable marker gene or genes or foreign ancillary nucleic acid from a plant and especially from a transgenic plant; means therefor and products thereof.

Background to the Invention

Recombinant genes conferring resistance to antibiotics or herbicides are widely used as selectable markers in plant transformation. Once transgenic material has been selected on the basis of its resistance, the marker gene is dispensable. The presence of antibiotic or herbicide resistance markers in transgenic plants has caused concern about a potential spread of resistance traits to wild plant relatives[1] or to other species[2].

The removal of resistance marker genes from plant transgenes is highly desirable for a number of reasons. Cross pollination between related species can lead to a transfer of resistance traits into weeds [3] jeopardising the long-term use of transgenic crops and causing potential ecological problems. For example, a marker encoding either antibiotic or herbicide resistance, could change a transgenic plant into a weedy pest which in turn could disrupt the ecosystem balance. Consumer groups express concern about a widespread distribution of resistance markers in food products, referring to the theoretical risk of a horizontal transfer of transgenes into gut bacteria. If this theoretical risk were to be realised, a marker gene could be transferred into microorganisms and increase the number of resistant pathogenic microorganisms in the human and/or animal gut.

A yet further reason for wanting to remove resistance marker genes from transgenic plants is that since there is only a limited number of selection marker genes that can be used for plant transformation, the combination of multiple transgenic traits via crosses among different transgenic lines will frequently produce plants that contain

multiple copies of the same selection marker linked to different effector genes. Since the presence of a particular marker gene in a transgenic plant precludes the use of that marker in subsequent transformations, it will be necessary to use different selectable marker systems for each transformation. Thus, in the long term there is likely to be more transgenes one may wish to introduce than the number of suitable selectable markers available. A further problem is that the presence of multiple homologous sequences in plants enhances the likelihood for homology-dependent gene silencing[4], which could severely limit the reliable long-term use of transgenic crops.

Given the foregoing reasons for desiring the removal of resistance markers from plant transgenes, a number of systems have been developed to ensure the removal of selectable marker genes. For example, co-transformation of two different constructs can result in transgenic lines that have integrated both transgenes [5] but the applicability of this approach is limited because it depends on the efficiency at which both transgenes insert into different genomic regions as this is a requirement to separate them in genetic crosses. Moreover this method has a further disadvantage in that it is laborious and time consuming because of the requirement of a genetic cross.

As an alternative to co-transformation, several transposable element systems and site-specific recombination systems have been employed for marker removal [6]. These systems require the expression of a transposase or recombinase that mediates the deletion of regions bracketed between recombination or transposase target sequences, and the subsequent removal of the helper gene via genetic segregation, which makes these systems relatively time consuming. Moreover, deleted fragments can reinsert into other genomic positions and recombinase or transposase proteins have the potential to cause undesirable secondary effects.

Another approach to removing selectable gene markers is to induce DNA deletions based on intrachromosomal homologous recombination (ICR) between two homologous sequences. Although ICR can be enhanced by stimulation of repair systems, the inherent drawback to ICR is that frequencies are too low for an efficient

application of this system to produce deletions of transgene regions. In tobacco, for example, on average less than ten ICR events are detectable among all cells of a six-week old tobacco plant [7].

Despite the foregoing limitations of the ICR method we have developed an ICR strategy based on recombination of a hitherto unexploited region of bacteriophage λ and we have been able to generate a surprisingly and sufficiently high efficiency of ICR that allows for a simplified identification of tissue that has lost a transgenic marker gene.

Statement of the Invention

According to a first aspect of the invention there is provided a method of removing a part of a transgene after its integration into a genome comprising flanking said part of the transgene on each side thereof with an attachment P region (attP) of bacteriophage λ and inducing intrachromosomal homologous recombination between each flanking attP regions whereby said part of a transgene sandwiched therebetween is removed.

Reference herein to attachment P region (attP) is intended to include a region of bacteriophage λ DNA that is associated with high recombination efficiency.

Preferably, said part of said transgene comprises a marker gene and/or vector sequence and/or other foreign ancillary nucleic acid.

Preferably, the genome is a plant genome.

Preferably, the marker gene confers resistance to antibiotics and/or herbicide resistance.

It will be appreciated that the term marker gene is intended to include genes involved in specific biosynthetic pathways and/or genes involved in environmental tolerance.

Preferably, the marker gene is selected from the group consisting of *nptII*, Ble, *dhfr*, *cat*, *aphIV*, SPT, *aacC3*, *aacC4*, *bar*, EPSP, *bxn*, *psbA*, *tfdA*, DHPS, AK, *sul*, *crs1-1* and *tdc*.

Preferably, the method is capable of deleting in the region of up to 10 kb between each of the two attP regions and more preferably in the region of 7kb.

Preferably, in the instance of removing more than one marker gene and/or vector sequence and/or other foreign ancillary nucleic acid each undesirable part of the transgene to be removed is flanked by att P regions. Thus it will be appreciated that the method of the invention can simultaneously be used to remove more than one undesired part of the genome at the same time.

Preferably, the attP region comprises 352bp located between position 27492 and 27844 of bacteriophage λ.

Preferably the attP region comprises the nucleic acid sequence as set forth in SEQ ID NO:1, or fragment thereof with the same functional equivalent, or nucleic acids which hybridise under stringent conditions to the DNA of SEQ ID NO:1 and function as an attP region, or nucleic acids which differ from the DNA of SEQ ID NO:1 due to the degeneracy of the genetic code and which function as an attP region.

The method of the invention provides a novel strategy to remove undesirable and/or other parts of a transgene after its integration into a plant genome. The method of the invention exploits the hitherto unrecognised potential of the high recombination efficiency of the attachment P region (attP) of bacteriophage λ, producing deletion events after intrachromosomal recombination between two attP regions. The attP system has been demonstrated to delete a 5.9kb region from a recombinant vector

that had been inserted into two different genomic regions. In contrast to other deletion systems that are based on bacterial recombination systems or on the use of transposable elements [6], the attP system does not require the expression of recombinant helper proteins to induce deletion events. Thus, the attP deletion method of the invention provides a simple and efficient tool to improve the potential of plant transformation and to address many of the problems including consumer concern about the use of resistance markers.

Preferably, the attP regions are in a cassette.

Preferably, the cassette further includes a transformation booster sequence (TBS) or fragment thereof for enhancing homologous and illegitimate recombination.

The TBS is typically derived from *Petunia hybrida*.

Preferably, the cassette further includes an effector gene such as oryzacystastin-I or functional equivalent thereof.

According to a further aspect of the invention there is provided a plant having been produced using the method as hereinbefore described so that a part integrated into its genome is removed and optionally further including any of the preferred features as hereinbefore described.

Preferably, the plant is grown and plant products harvested therefrom.

According to a yet further aspect of the invention there is provided a plant cell having been produced using the method as hereinbefore described so that a part integrated into its genome is removed and optionally further including any of the preferred features as hereinbefore described.

Preferably, the plant cell is grown and plant products harvested therefrom.

According to a yet further aspect of the invention there is provided an attP recombination cassette comprising a marker gene and/or vector sequence and/or other foreign ancillary nucleic acid flanked on each side by an attP region.

Preferably, the cassette further includes any of the preferred features as hereinbefore described.

According to a yet further aspect of the invention there is provided a plant or plant cell or plant tissue comprising recombinant attP regions. In addition the invention also includes seeds.

According to a yet further aspect of the invention there is provided use of an attP recombination cassette for removing a part integrated into a plant genome and optionally further including any of the preferred features as hereinbefore described.

According to a yet further aspect of the invention there is provided a kit for removing a part of a transgene after its integration into a plant genome comprising an attP recombination cassette as herein before described and optionally including any of the preferred features as hereinbefore described.

According to a yet further aspect of the invention there is provided a plant or plant cell or plant tissue having a recombinant transgene integrated into its genome the transgene being associated with a bacteriophage λ attP region.

Preferably the plant or plant cell or plant tissue includes at least one λ attP region and one effector trangene integrated into its genome. It will be appreciated that the plant or plant cell or plant tissue ideally comprises at least two attP regions to effect ICR but that the plant or plant cell or plant tissue can comprise a first attP region and a second attP region can be subsequently introduced so as to flank the transgene.

Preferably the plant or plant cell or plant tissue is characterised in that the λ attP region and one transgene are not associated with a marker gene and/or vector sequence and/or other foreign ancillary nucleic acid.

Preferably the transgene is further associated with a transformation booster sequence or fragment thereof which is capable of enhancing homologous and illegitimate recombination.

Detailed description of the Invention

The invention will now be described, by way of example only with reference to the following Figures wherein:

Figure 1 represents T-DNA region of pattP-ICR;

Figure 2 represents selection of marker-free transgenic tobacco plants;

Figure 3 illustrates PCR analysis of the km-resistant and km-sensitive plants and includes the nucleic acid sequence of the λ bacteriophage attP region (SEQ ID NO:1); and

Figure 4 illustrates Southern blot analysis of the km-resistant and km-sensitive plants.

Figure 1 represents T-DNA region of pattP-ICR. Two 352 bp regions of the attP region (located between position 27492 and 27844 of the phage λ genome) were inserted at both sides of the NPTII gene in vector pPCV002 [8]. Into the attP cassette, we inserted the tms-2 coding region and polyA region [9] and the GFP coding region [10] linked to the nos polyA region. Both genes are transcribed by the dual 1'-2' promoter [11]. The attP cassette was embedded into an 0.6 kb DS element that would allow removal of the complete cassette in the presence of an active Ac transposase 5'

to the attP cassette a TBS fragment was inserted that enhances illegitimate and homologous recombination and an oryzacystatin-I gene as an example for an effector gene. Arrows indicate the regions amplified by the primers specific for detection of the effector gene (PE1 and PE2), the NPTII gene (PN1 and PN2) and the attP cassette (P5' and P3'). ICR between the two attP regions will generate a 5.9kb deletion with only one attP region remaining and the region between the attP regions being removed. Black arrows indicate probes used in Southern blot hybridisation (Figure 4).

Figure 2 represents selection of marker-free transgenic tobacco plants (A). Lines 1 and 2 develop green and white shoots on km-containing medium. White tissue, which has potentially lost the NPTII marker, was further tested for activity of the tms-2 gene (B) On NAM-containing medium shoots with tms2 activity produce abundant calli instead of roots (left), while shoots regenerated from white tissue that have lost the tms2 gene produce normal roots (right).

Figure 3 illustrates PCR analysis of the km-resistant plants derived from line 1 (lane 2) and line 2 (lane 4), and km-sensitive plants derived from white tissue of line 1 (lane 3) and line 2 (lane 5). Lane 1 contains a *Hind*III-digested λ DNA as size marker. Sizes of PCR fragments are indicated in bp.

A. PCR with primers PE1 and PE2 shows that all four lines contain the effector gene.

B. PCR with primers PN1 and PN2 shows that the km-sensitive plants have lost the NPTII gene.

C. PCR with primers P5' and P3' show that the two km-resistant lines contain the complete attP cassette, while in both km-sensitive plants about 6kb have been deleted from the attP cassette.

D. Sequence of the PCR product shown in C, lane 3, confirms that recombination leaves precisely one attP region bracketed by the 5' and 3' regions.

Figure 4 illustrates Southern blot analysis of DNA isolated from km-resistant (lane 2) and km-sensitive tissue of line 1 (lane 3).

A. Genomic DNA digested with ScaI was probed with region "a" (see Figure 1) to label the left junction fragment of the integrated T-DNA.

B. Genomic SDNA digested with ScaI was probed with region "b" (see Figure 1) to label the right junction fragment of the integrated T-DNA.

In km-sensitive tissue, the left end fragment remains unaltered (A) while the right end fragment is shortened by about 6kb due to the deletion of the region between the two attP regions (B).

Materials and Methods

Plant transformation

The pattP-ICR construct was introduced into A. tumefaciens strain GV3101 (pMP90RK) by conjugation as described by Koncz et al.[12]. Leaf disc transformation of Nicotiana tabacum cv. Petit Havana SR1 was performed according to Horsch et al.[13].

DNA preparation and analysis

After DNA mini preparation [14], PCRs were performed in a reaction volume of 50 µl containing 10 mM TRIS-HCl PH 8.3, 50 mM KCL, 2mM $MgCL_2$, 0.1% w/v gelatin, 0.2 mM of each nucleotide, 25 pmol of each primer and 1U of Taq polymerase (Promega). The PCR cycles used were, for detection of the effector gene: 4 min at 94°C, 30 cycles of 1 min at 94°C, 30 sec at 55°C, 30 sec at 72°C followed by 10 min at 72°C , and to amplify the sequence of NPT II gene: 4 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C followed by 10 min at 72°C. For amplification of the attP cassette, a long template PCR was performed using the Expand™ Long Template PCR System according to manufacturer's protocol (Boehringer Mannheim). The sequences of the primers used in PCR were:

PE1: TCA TCA GAC GGA GGA CCA GTT TTG G (SEQ ID NO:2)
PE2: ATC CAT GGT TTT TCC CAA ACT TTA G (SEQ ID NO:3)
PN1: CCA TGA TCA TGT CGA TTG AAC AAG ATG (SEQ ID NO:4)
PN2: CCA TTT TCC ACC ATG ATA TTC GGC AAG (SEQ ID NO:5)
P5': GAA TTC TAA TTC GGG ATG ACT GCA ATA TGG (SEQ ID NO:6)
P3': GGA TCC AAC GGG ATA TAC CGG TAA CGA AAA CG (SEQ ID NO:7)

Southern blot analysis

Genomic DNA was isolated as described by van Blokland et al. [15]. Fifteen μg of genomic DNA was digested with Sca I and fractionated by electrophoresis in 0,7% agarose gel. After electrophoresis the DNA was blotted on Nybond N filter, crosslinked by UV irradiation and hybridized with a ^{32}P-labelled 0,3kb Eco RI/Sac I DNA fragment of the effector gene at 65°C according to Koes et al. [16].

Results

Integration of bacteriophage λ into the *E. coli* genome occurs via recombination between the phage attachment region (attP) and the bacterial attachment region (attB). The bacterial integration host factor (IHF), and the virally encoded integrates (Int) gene are required for attP integration, while excision requires in addition the viral excisase (Xis) protein. The three recombination proteins bind to defined DNA regions within a 250bp attP fragment [17]. IHF fulfils an accessory function, bending the attP region [18] and possibly assisting Int in organising attP into a nucleosome-like structure that is required for efficient synapsis with attB [19]. The actual strand exchange, which occurs within a 7bp homology between the core regions of attP and attB is mediated by the Int protein that has a toposisomerase I activity. A tyrosine residue of Int covalently binds to the 3' end of the DNA, resembling a mammalian topoisomerase I type in contrast to *E. coli* toposisomerases I that binds to the 5' end of the DNA[20].

In the present invention we have designed and constructed a plant transformation vector pattP-ICR, in which we inserted two 352bp attP regions so as to flank an NPTII resistance marker, a GFP gene and a tms2 gene (figure 1). Next to one attP site, we positioned the Transformation Booster Sequence (TBS) that enhances homologous and illegitimate recombination [21] and an orystastatin-I gene [22] that served as an example for an 'effector' gene that would finally be transferred into the genome via the attP system. ICR between the two attP regions should delete the 5.9 kb region between the attP regions and produce a transgene that retains the effector gene and the TBS sequence. The construct was introduced into tobacco via leaf disk transformation and resistant calli were selected on kanamycin (km) medium. Two months after transformation, eleven km-resistant calli, 0.5 cm in diameter, were transferred to km-free medium. When calli had grown to 5-6 cm in diameter, they were cut into smaller parts that were transferred onto km-containing shoot regeneration medium. After 3-5 months, multiple shoots had developed on all eleven calli, we detected two clones that produced a mixture of green and white shoots. These two clones (line 1 and 2, figure 2) were further studied to evaluate whether white shoots contained tissue that had lost the NPTII resistance marker and the tms2 gene.

White leaves of both lines were placed on km-free regeneration medium and regenerating shoots were placed on medium supplemented by Naphtaleneacedamide (NAM). As tms2 gene activity converts NAM into the auxine NAA [9], plants expressing the tms2 gene produce high auxine levels that prevent root development and induce callus production instead. Tissue that had deleted the region between the two attP fragments should have also lost the tms2 gene and can therefore be identified due to its ability to form roots on NAM containing medium. Eleven out of 20 shoots tested for line 1 and twelve out of 32 shoots tested for line 2 produced roots, and these 23 plantlets were further analysed as potential candidates that had lost both the NPTII gene and the tms2 gene. PCR analysis showed that all 23 plantlets had indeed lost the NPTII gene and the tms2 gene. Two of the eleven line 1

derivatives and one of the twelve line 2 derivatives had retained the effector gene, while all other lines had lost the effector gene as well.

One representative plant from line 1 and line 2 that had retained the effector gene was further characterised to analyse the precise degree of the deletion events. As a control, we used km-resistant shoots of lines 1 and 2. PCR analysis with primers specific for the effector gene, the NPTII gene and the region flanking the attP dimer cassette showed that the effector gene had been retained, while the NPTII gene had been lost and the region between the two attP fragments had been reduced by about 6 kb, as expected if the two attP sites had recombined (Figure 3). Both clones produced identical PCR patterns indicating that they had both generated the same deletion product. Sequencing of one of the PCR fragments confirmed that precisely one attP region had been maintained between the 5' and 3' region flanking the attP cassette, which is in accordance with the expectation that ICR between the two attP fragments has deleted the 5.9kb region between them. A Southern blot analysis of green and white tissue of line 1 confirmed the PCR data (Figure 4).

The fact that most plantlets that have lost the NPTII/tms2 region also have lost transgene regions outside the attP cassette, shows that ICR is not always associated with precise homologous recombination between the two attP regions but that it can generate larger deletion probably due to illegitimate recombination. However, ICR events can clearly be identified where precise intrachromosomal recombination had occurred between the two attP regions. As the same deletion event can be found in two different transformants, it is unlikely that efficient recombination between attP regions only occurs within transgenes that are integrated at specific genomic integration regions. Our data shows that, starting with a relatively small number of calli, homogeneous material with precise deletion events can be selected, and that, due to the availability of PCR technology, such events can easily be distinguished from undesirable deletion events that have lost more than the region between the two attP sites. As at least 35% of the shoots regenerated from white tissue represent

deletion events, the use of the tms2 gene for preselection of deletion events appears dispensable and can probably also be substituted by PCR.

The reason for the relatively high ICR efficiency of the attP fragment is unclear but it is tempting to speculate that this effect is due to an improved accessibility of the attP regions. Somatic intrachromosomal recombination in tobacco is part of a repair mechanism that is stimulated by DNA damage [7]. The efficiency of ICR in plants is normally several magnitudes lower than the efficiency of extrachromosomal recombination suggesting that the packaging of DNA into chromatin significantly protects it from the repair/recombination apparatus [23]. Improved accessibility of DNA regions due to an altered chromatin structure could therefore have a drastic effect on the efficiency of ICR. Support for this assumption comes from experiments in human fibroblasts demonstrating an enhanced DNA repair synthesis in hyperacetylated nucleosomes as these have a more open chromatin structure than hypoacetylated nucleosomes [24].

The attP fragment has a relatively high AT content (67%) that might be responsible for its potentially improved accessibility. AT rich regions have been identified as preferential target site for transgene integration into the plants genome mediated by illegitimate recombination, and it has been proposed that a high AT content increases the probability of DNA integration through steric bending of the chromosomal DNA structure[25]. As a working hypothesis to explain the high recombination efficiency of attP sites, we propose that the attP region, due to its sequence composition alone or in combination with nuclear proteins associated with the attP region undergoes a conformational change that improves its accessibility for repair/recombination enzymes. In bacteria, bending of the attP region is mediated by IHF, a small basic proteins composed of two dissimilar subunits. Both subunits show features of histone-like bacterial proteins [26], and one could speculate that an interaction of the attP region with plant histones or histone-like proteins may equally lead to a bend structure.

While the molecular mechanisms responsible for the high ICR efficiency of the attP regions remain to be determined, the exploitation of this unexpected effect should provide a useful tool for rapid and efficient removal of resistance marker genes or any other undesirable transgene regions from transgenic plants.

5

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